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The putative tumor suppressor gene EphA3 fails to demonstrate a crucial role in murine lung tumorigenesis or morphogenesis

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ABSTRACT
Treatment of non-small cell lung cancer (NSCLC) is based on histological analysis and molecular profiling of targetable driver oncogenes. Therapeutic responses are further defined by the landscape of passenger mutations, or loss of tumor suppressor genes. We report here a thorough study to address the physiological role of the putative lung cancer tumor suppressor Eph receptor A3 (EPHA3), a gene that is frequently mutated in human lung adenocarcinomas. Our data shows that homozygous or heterozygous loss of EphA3 does not alter the progression of murine adenocarcinomas that result from Kras mutation or loss of Tp53, and we detected negligible postnatal expression of EphA3 in adult wild-type lungs. Yet, EphA3 was expressed in the distal mesenchyme of developing mouse lungs, neighboring the epithelial expression of its Efna1 ligand; this is consistent with the known roles of EPH receptors in embryonic development. However, the partial loss of EphA3 leads only to subtle changes in epithelial Nkx2-1, endothelial Cd31 and mesenchymal Fgf10 RNA expression levels, and no macroscopic phenotypic effects on lung epithelial branching, mesenchymal cell proliferation, or abundance and localization of Cd31-positive endothelia. The lack of a discernible lung phenotype in EphA3-null mice might indicate lack of an overt role for EPHA3 in the murine lung, or imply functional redundancy between EPHA receptors. Our study shows how biological complexity can challenge functional validation of mutations identified in sequencing efforts, and provides an incentive for the design of knock-in or conditional models to assign the role of EPHA3 mutation during lung tumorigenesis.

KEY WORDS: EPHA3, EPH receptor A3, GEMM, Adenocarcinoma, Lung morphogenesis

INTRODUCTION
Lung cancer is a leading cause of cancer-related deaths worldwide. More than 85% of all lung cancers are classified as non-small cell lung cancer (NSCLC), which is further sub-classified as adenocarcinoma (ADC; ~50%) and squamous cell carcinoma (SCC; ~40%) (Chen et al., 2014). In recent years, excellent progress in molecular profiling of NSCLC has identified stratified patient groups that benefit from targeted therapies (Oxnard et al., 2013). Specifically, erlotinib or gefitinib are prescribed to patients that carry mutations in epidermal growth factor receptor (EGFR), and crizotinib to carriers of anaplastic lymphoma kinase (ALK) gene rearrangements. However, despite an increase in progression-free survival, the overall survival benefit of such tyrosine kinase inhibitors remains marginal, and profound intra- and inter-tumor genetic heterogeneity confounds effective long-term responses (de Bruin et al., 2014).

Next-generation sequencing of lung cancer patient tumors has identified numerous putative new cancer drivers, including EPH (also defined as erythropoietin-producing hepatocellular) receptor A3 (EPHA3), which is mutated in 6–16% of lung ADC samples (Cancer Genome Atlas Research Network, 2014; Ding et al., 2008; Imielinski et al., 2012). The EPH receptors make up the largest family of receptor tyrosine kinases (RTKs) and, together with their ephrin ligands, they control a variety of biological processes. They are classified into two subclasses based on sequence homologies, namely EPHA and EPHB receptors and their ephrin-A and ephrin-B ligands. Interaction between the EPH receptors and their ligands at cell-cell contacts triggers signaling into both the receptor- and ligand-expressing cell. Such bidirectional signaling induces changes in the actin cytoskeleton, cell-substrate adhesion, intercellular junctions and cell shape, impinging on cell movement and tissue patterning (Pasquale, 2010). Context-dependent cellular responses are finely tuned by the abundance and type of receptor-ligand pairs expressed in neighboring cells, leading to specialized cell functions known to control synaptic plasticity, insulin secretion, epithelial homeostasis and inflammatory immune responses (Gucciardo et al., 2014; Pasquale, 2010).

The expression pattern of EphA3 in mammalian tissues suggests that there is a role for EPHA3 in neuronal development and formation of mesoderm-derived tissues (Kilpatrick et al., 1996; Kudo et al., 2005; Yue et al., 1999). However, in contrast to predictions made based on its expression in the developing medial motor column, constitutive loss of murine EphA3 does not lead to abnormal motor axon topography (Vaidya et al., 2003). Instead, 75% of the null mice die at birth owing to cardiac abnormalities caused by defective endothelial-to-mesenchymal transition, a specific form of mesenchymal conversion that generates progenitors of the atrioventricular valves (Stephen et al., 2007).

With respect to its putative role in tumorigenesis, previous studies have indicated that EPHA3 can signal both in a kinase-dependent and kinase-independent manner, inducing both tumor-promoting and tumor-suppressing effects (Boyd et al., 2014). For example, in glioblastoma multiforme, EPHA3 is highly expressed in undifferentiated mesenchymal cells where it has been shown to confer a kinase-independent oncogenic role through regulating mitogen-activated protein kinase (MAPK) signaling (Day et al., 2013). A tumor-suppressive role of EPHA3, in particular for lung cancer, is supported by the reduction in receptor activity conferred by the point mutations found in cancers, and ligand- and EPHA3-
dependent apoptosis of tumor and stroma cells upon receptor agonist treatment, suggesting that wild-type EphA3 has anti-tumorigenic properties (Lahtela et al., 2013; Lisabeth et al., 2012; Vail et al., 2012; Zhuang et al., 2012). Furthermore, the finding that senescence elicited by acute EphA3 loss is rescued by loss of p16INK4A (encoded by Cdkn2a) or p53 (encoded by Trp53) suggests that EphA3 mutation might promote tumorigenesis only in the absence of senescence-inducing pathways (Lahtela et al., 2013). Given the opposing outcomes of aberrant EPHE-ephrin signaling, careful dissection of the tissue and cell-context-specific EPHE receptor functions requires studies that utilize valid in vivo model systems.

Genetically engineered mouse models (GEMMs) are the most widely applied and functionally validated in vivo models of human lung cancer, in particular to validate gene cooperation concomitant with conditional expression of the oncogenic Kras gene (Jackson et al., 2001; Jackson et al., 2012; Ji et al., 2007; Schramek et al., 2011; Snyder et al., 2013). Importantly, murine clinical studies have shown that oncogenic signaling in Kras-driven GEMMs is crudely defined by the cooperating tumor suppressor, with loss of liver kinase B1 (Lkb1) conferring different therapeutic responses compared with loss of Trp53 (Chen et al., 2012). Despite convincing data suggesting a tumor suppressor role for EphA3 during lung tumor progression, thus far no studies have addressed its in vivo functional role. We therefore decided to utilize the EphA3-null mice to test the effect of constitutive loss of EphA3 on lung ADC progression driven by mutant Kras (LSL-KrasG12D) (Jackson et al., 2001) and loss of Trp53 (p53fl/fl) (Marino et al., 2000), hereafter referred to as Kras and p53. Our data shows that the constitutive loss of EphA3 does not alter the progression of murine ADC in either of these models. Moreover, despite clear evidence for EphA3 expression in the developing lung, similar to key regulators of morphogenesis known to regulate lung tumorigenesis (Clark et al., 2001; Snyder et al., 2013; Yin et al., 2013), an analysis of selected EphA family receptors shows that EphA3 has a non-unique or minimal function during lung morphogenesis. Our study thus provides an incentive for rational design of novel GEMMs to unequivocally assign the role of EphA3 during lung tumorigenesis in vivo.

RESULTS

Constitutive loss of EphA3 does not accelerate mutant Kras- or p53-loss-driven lung tumorigenesis

To test the hypothesis that EphA3 acts as a lung tumor suppressor, we used a previously described constitutive EphA3-null mouse model (Stephen et al., 2007; Vaidya et al., 2003). EphA3-null mice did not show any marks of reduced survival during a 1-year follow-up period, indicating that mere EphA3 loss does not drive tumorigenesis. We therefore assessed whether EphA3 loss could accelerate tumorigenesis induced by conditional alleles known to initiate lung ADC, following a classic multi-allele paradigm. These ‘first hit’ conditional models comprised mutant Kras (Jackson et al., 2001) and loss of p53 (Marino et al., 2000), which are also common drivers of human disease found in at 17% and 35% of ADCs, respectively (COSMIC, 2014; http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/). In lung ADC, EphA3 mutations show a statistically significant tendency towards co-occurrence with mutations in TP53 (P<0.01) and occasional, but not statistically significant, co-occurrence with KRAS mutations (supplementary material Fig. S1A). We established cohorts of 8-16 mice for each genetic combination (homozygous p53 or heterozygous KRAS with wild-type EphA3, or homozygous or heterozygous null EphA3). Lung-specific deletion of conditional alleles was achieved through intranasal inhalation of adenoviral Cre recombinase (CMV-AdCre), affording transduction of bronchiolar and alveolar progenitor cells, to initiate carcinoma progression. The infection efficacy was confirmed with a dual fluorescence mT/mG Cre-reporter strain that monitors in vivo integration efficiency through activating a CRE-dependent switch from membrane-tagged Tomato to GFP (supplementary material Fig. S1B) (Muzumdar et al., 2007). Tumor burden analysis at 19 weeks after CMV-AdCre infection showed that constitutive loss of EphA3 did not alter mutant Kras-driven lung ADC progression (Fig. 1A,B; supplementary material Fig. S1C). Similar to previous findings in murine Kras lung cancer studies (Jackson et al., 2001), all EphA3 genotype cohorts displayed distinct types of progressive lesions, including epithelial hyperplasia, adenomas and ADCs. In addition, we detected previously described profound inflammatory responses as infiltrations of macrophages and neutrophils (supplementary material Fig. S1C) (Ji et al., 2006). Furthermore, analysis of histopathology and NKX2-1 and tumor protein 63 (p63) biomarker expression to respectively depict ADC and SCC tissue, showed that constitutive absence of EphA3 did not alter the tumor histology (Fig. 1C). We further found that the loss of EphA3 did not alter the latency of p53-loss-driven ADCs (Fig. 1D). Thus, the constitutive absence of EphA3 expression does not accelerate mutant Kras- or p53-loss-driven lung tumorigenesis.

Mesenchymal expression of EphA3 suggests that it has a functional role during lung development

As previous studies have indicated a role for EphA3 in embryonic development, we undertook a detailed expression analysis of EphA3 during lung tumorigenesis.
in the developing mouse lung. Both RNA in situ hybridization (Fig. 2A) and immunohistochemical staining (Fig. 2B) demonstrated expression of EphA3 in the distal mesenchyme of the embryonic lung. The specificity of the EPHA3 antibody was confirmed by absence of detectable immunohistochemical staining in EphA3-null embryos (Fig. 2A,B), as well as a decreased signal in hTERT-RPE1 cells treated with EPHA3 small interfering RNA (siRNA) (supplementary material Fig. S1D). Expression of EphA3 in the developing lung was detected during embryonic ages E11.5 to E15.5 (Fig. 2B,C), which falls into the pseudoglandular (E9.5-E16.5) stage of murine lung development. During this stage, the newly generated primary lung buds develop into a complex branched tree-like structure ending in thousands of epithelial terminal tubules, accompanied by continued mesenchymal growth around the growing epithelia (Morrisey and Hogan, 2010). Based on this mesenchymal expression in the developing mouse lungs we hypothesized that EPHA3 might function during the pseudoglandular stage of lung development.

Expression of multiple EPH receptors in the developing mouse lung suggests involvement in lung morphogenesis

We next asked how mRNA expression of EphA3 during murine lung morphogenesis might correlate with or impact on mRNA expression of other EphA receptors in epithelial and mesenchymal cells at E11.5, E13.5 and E15.5 of lung development (Fig. 3A). At E11.5 we performed quantitative PCR (q-PCR) expression analysis on both proximal and distal epithelium and mesenchyme. At E13.5 and E15.5, the analysis was restricted to the distal regions, approximating the terminal epithelial buds and their surrounding mesenchyme. We found that among the studied EphA receptors, only expression of EphA3 was restricted to the developing lung mesenchyme, and closely overlapped with expression of known mesenchymal Fgf10 and endothelial Cd31 (also known as Pecam1) genes (Fig. 3B; supplementary material Fig. S2A). EphA7 expression was detected both in the mesenchyme and epithelia, and was the only other EphA receptor co-expressed with EphA3 in the mesenchyme (Fig. 3B). Importantly, we did not detect any
compensatory changes in EphA7 expression levels in the heterozygous or homozygous EphA3-null embryonic lungs (supplementary material Fig. S2B). EphA2 and EphA4 were found to be expressed mainly in epithelial cells, whereas expression of EphA1 and EphA5 was absent in both tissue compartments (Fig. 3B). These results correlate with in situ hybridization data described by the Allen Institute for Brain Science, with the exception of EphA1, for which moderate expression was detected in murine lung epithelia (Allen Institute for Brain Science, 2013). We further confirmed the epithelial expression of the known ligand of EPHA3, ephrin-A1 (encoded by Efna1), at E11.5, E13.5 and E15.5 (Fig. 3B; supplementary material Fig. S2C). Finally, postnatal murine lung expression analysis revealed very low EphA3 and EphA7 expression levels when compared to that of the embryonic mesenchyme (E13.5), whereas EphA2 and EphA4 expression were higher in the adult tissue (supplementary material Fig. S3A). Taken together, our data identifies EphA3 as a mesenchymal EPH receptor and suggests that its ligand ephrin-A1 is expressed in the adjacent branching epithelia. Furthermore, the low expression of EphA3 in adult tissue suggests that EPHA3 function might modulate lung morphogenesis.

EphA3 heterozygosity is associated with altered expression of branching morphogenesis and vasculogenesis genes

Next, we investigated whether constitutive loss of EphA3 affected the mRNA expression of known lung morphogenesis genes. A targeted q-PCR analysis of known regulators of lung morphogenesis identified a small but significant increase in the expression of Nkx2-1 in heterozygous EphA3 embryonic epithelium at E13.5 when compared with wild-type tissue (Fig. 3C). Furthermore, similar expression increases were detected in endothelial Cd31 and mesenchymal Fgf10 (Fig. 3C). In contrast, analysis at E15.5 failed to show statistically significant expression differences for these three genes (supplementary material Fig. S3B) suggesting that any role for EphA3 during pseudoglandular lung development is transitory. Taken together, the partial loss of EphA3 appears to induce subtle and transitory alterations in epithelial Nkx2-1, endothelial Cd31 and mesenchymal Fgf10 mRNA expression, suggesting that EPHA3 function might modulate lung morphogenesis.

Constitutive loss of EphA3 does not overtly affect murine lung morphogenesis

We next asked whether the constitutive loss of EphA3 was directly associated with altered lung branching morphogenesis. We first performed a quantitative analysis of lung branch end-points at E13.5 by E-cadherin whole-mount immunohistochemistry staining and optical projection tomography (OPT) to visualize branching epithelia. The number of terminal branches was found to be identical in EphA3 heterozygous (average 113) and null embryonic lungs (average 110) when compared to age-matched littermate controls (average 108) (Fig. 4A,B). Additional qualitative analysis using E-cadherin-stained E11.5 and E15.5 whole-mount lungs further confirmed that EPHA3 does not overtly affect lung branching morphogenesis (supplementary material Fig. S3C). Next, we assessed whether loss of EphA3 was associated with an alteration in distal mesenchymal cell proliferation. Analysis of in vivo BrdU incorporation showed that there was no statistically significant increase in the percentage of mesenchymal S phase cells in EphA3 heterozygous (36%) or EphA3-null (26%) lungs at E13.5 when compared to littermate controls (26%) (Fig. 4C). Finally, we studied whether the pulmonary vasculature formation was altered by loss of EphA3 by analyzing CD31 expression at E13.5. In both EphA3-null
and heterozygous lungs, the number of CD31-positive endothelial cells at E13.5 was identical to that of the controls (Fig. 4D). Taken together, the data presented here show that constitutive loss of EphA3 does not overtly alter murine lung morphogenesis.

**DISCUSSION**

The functional validation of *de novo* mutations identified in lung cancer sequencing efforts is a prerequisite for the development of novel targeted therapies. *EPHA3* is among the most frequently mutated RTKs in human lung ADCs, and has been assigned a candidate tumor suppressor role based on its mutation spectrum and findings from *in vitro* and *in vivo* studies (Lahtela et al., 2013; Lisabeth et al., 2012; Vail et al., 2014; Zhuang et al., 2012). However, the actual role of EPHA3 during lung tumor progression has not been investigated nor validated using GEMMs. Our previous findings linked loss of EPHA3 to p53 activation (Lahtela et al., 2013), and *EPHA3* and *TP53* point mutations display statistically significant co-occurrence in lung ADC (supplementary material Fig. S1A). We hence asked whether the absence of *EphA3* enhanced the incidence of p53-loss-driven lung cancer progression. Additionally, we asked whether loss of *EphA3* accelerated lung ADC progression caused by the commonly mutated *Kras* oncogene. We here show that the constitutive absence of EPHA3 does not affect tumor progression and histopathology of both p53-loss- and mutant *Kras*-driven lung ADCs. Thus, *EphA3*-null mice fail to validate a putative tumor suppressor function for *EPHA3* in human lung cancer, perhaps owing to functional redundancy between murine EphA receptors expressed in adult lungs. Interestingly, sequencing of murine small cell lung cancer (SCLC) tumors initiated by loss of p53 and retinoblastoma 1 (Rb1) revealed that there were recurrent somatically acquired *EphA5* and *EphA7* mutations (McFadden et al., 2014). This means that there is a strong case for further lung tumorigenesis studies to study the role of EphA receptor biology in GEMMs, and in particular the physiological role of *EphA5* and *EphA7*.

Re-activation of EPH-receptor–ephrin pathways, generally known to contribute to cell sorting and tissue patterning in embryonic development, has been causally linked with tumorigenesis
Moreover, expression of key regulators of embryonic lung morphogenesis, *Fgf9* and *Fgf10* (Colvin et al., 2001; Min et al., 1998; White et al., 2006), has been shown to trigger ADC and adenoma progression, respectively (Clark et al., 2001; Yin et al., 2013). Thus far, of all EphA receptors and ligands, only a role of the ephrin-B2 ligand has been described during lung development. Specifically, ephrin-B2 has been shown to regulate alveolar epithelial and endothelial viability and vascular growth in hyperoxic rats (Vadivel et al., 2012), as well as pulmonary compliance in mice (Bennett et al., 2013). Our current data shows that *EphA3* is expressed specifically in the mesenchymal distal lung tips during the pseudoglandular stage of branching morphogenesis,
albeit at low levels. However, whereas for example the removal of Fgf10 results in dramatic defects in lung organogenesis (Min et al., 1998), partial loss of EphA3 appears to induce only subtle increases in epithelial Nks2-1, endothelial Cd31 and mesenchymal Fgf10 mRNA expression levels. Furthermore, no macroscopic phenotypic effect on lung epithelial branching, mesenchymal cell proliferation, or abundance and localization of CD31-positive endothelia was measured. This lack of a discernible phenotype might indicate: (1) lack of an overt, or a different, role for EphA3 in the murine lung; (2) functional redundancy between lung-expressed EphA receptors; or (3) a partial penetrance of the EphA3-null genotype.

Of the selected EphA receptors, we found that only EphA7 was co-expressed with EphA3 in the lung mesenchyme. Interestingly, a recent study has suggested that there is functional compensation of EphA3 loss by EphA7 co-expression during palate development, as compound homozygous mutation of EphA3 and EphA4 failed to cause defective midfacial development (Agrawal et al., 2014).

Furthermore, a truncated form of EphA7 has been reported to act cause defective midfacial development (Agrawal et al., 2014).

In the applied constitutive null GEMM, Importantly, we cannot exclude the possibility that other EphA receptors co-expressed in the (developing) lung, most notably EphA7, can compensate for the decreased expression of EphA3. Our findings therefore provide an incentive to perform a rational design of tissue-specific knock-in or conditional mouse models to unequivocally assign the role of EPHA3 mutation or loss of expression, possibly in the context of compound EPHA-ephrin network mutations, on lung tumorigenesis in vivo. In this respect, the ability to apply prokaryotic type II CRISPR/Cas genome editing tools to introduce somatic tumorigenesis in epithelial specific knock-in or conditional mouse models to unequivocally compensate for the decreased expression of EphA receptors expressed in the (developing) lung, most notably EphA7, may provide for future tumor modeling approaches.

**MATERIALS AND METHODS**

**Mouse cohorts and tissue preparation**

Animal studies were carried out in accordance with guidelines from the Finnish National Board of Animal Experimentation, and were approved by the Experimental Animal Committee of the University of Helsinki and the State Provincial Office of Southern Finland (License number EASAV-2010-04855/Ym-23). EphA3-null mice lacking a genetic region encompassing the first exon of EphA3 were previously described (Stephen et al., 2007; Vaidya et al., 2003). Mice carrying a conditional mutant allele of Kras (LSL-KrasG12D) (Jackson et al., 2001) or a loss-of-function allele of Tp53 (p53<sup>−/−</sup>) (Marino et al., 2000) were purchased from The Jackson Laboratory. EphA3-null mice were bred with Kras and p53 mice to generate the study cohorts, and were maintained on a mixed genetic background using littermates as controls. Multiple litters of the same age were used to provide sufficient numbers of each genotype. Lung tumorigenesis was initiated by infecting mice at 6-10 weeks of age with 3.3×10<sup>7</sup> plaque-forming units (PFUs) of recombinant adenovirus expressing the Cre recombinase (University of Turku, Finland), using intranasal administration. The mouse EphA3 probe was an 817-bp fragment (nucleotides 658-1474) inserted into pGEM-3Zf- vector. The mouse Efa1 probe was a 402-bp fragment (nucleotides 20-421) inserted into pGEM-3Zf- vector. The Fgf10 probe was a 584-bp fragment (nucleotides 11-579) inserted into Bluescript KSII+ vector.

**BrdU proliferation assay**

A timed pregnant mouse was injected with 5-bromo-2-deoxyuridine (BrdU) (Sigma, St Louis, MO) and killed 4 hours later to harvest embryos at embryonic age of 13.5. Embryos were fixed in 4% formaldehyde and embedded in paraffin. Sections (4 μm) were cut from two distinct zones of the embryonic lungs. BrdU-positive cells were detected using anti-BrdU antibody (cell Signaling Technology, Danvers, MA) and counterstained with Hoechst 33342 dye (Invitrogen/Thermo Fisher Scientific Inc., Waltham, MA). Image acquisition was performed either using a Nikon 90i Eclipse microscope (Nikon Instruments Europe BV, The Netherlands) and DS-FI2 5 MP camera, or a Panomaromic 250 3DHISTECH (3DHISTECH Kft., Budapest, Hungary) digital slide scanner with a 20× objective.

**Histology and immunohistochemistry**

Immunohistochemistry was performed on paraffin-embedded sections (4 μm). Sections were dehydrated and antigenic epitopes were exposed by heating in 10-mM citrate buffer (pH 6.0) or by incubation in 0.05% trypsin at +37°C. Sections were incubated with the following antibodies: anti-NKX2-1 (Abcam, Cambridge, UK); anti-p63 (Abcam, Cambridge, UK); anti-EphA3 (Invitrogen/Thermo Fisher Scientific Inc., Waltham, MA); anti-CD31 (Becton, Dickinson and Company, Franklin Lakes, NJ); anti-GFP (polyclonal rabbit serum 8 mg/ml, generated in house). Primary antibody staining was detected using a peroxidase (HRP)-conjugated anti-rabbit rabbit IgG (Immunologic, Duiven, The Netherlands), HRP-conjugated anti-rabbit IgG (Life Technologies/Thermo Fisher Scientific Inc., Waltham, MA) and 3,3′-diaminobenzidine (DAB) (Immunologic, Duiven, The Netherlands) or Alexa-Fluor-488-conjugated anti-rabbit IgG (Life Technologies/Thermo Fisher Scientific Inc., Waltham, MA). Image acquisition was done using a Nikon 90i Eclipse microscope (Nikon Instruments Europe BV, The Netherlands) and DS-FI2 5 MP camera, or a Panomaromic 250 3DHISTECH (3DHISTECH Kft., Budapest, Hungary) digital slide scanner with a 20× objective.

**In situ hybridization**

Radioactive in situ hybridization was performed on paraffin sections according to the standard protocols using probes labeled with [35]<sup>S</sup>UTP. Dark-field images were inverted, linearly thresholded and combined with brightfield images in Adobe Photoshop CS6 (Adobe Systems Software, Dublin, Ireland). The mouse EphA3 probe was an 817-bp fragment (nucleotides 658-1474) inserted into pGEM-3Zf- vector. The mouse Efa1 probe was a 402-bp fragment (nucleotides 20-421) inserted into pGEM-3Zf- vector. The Fgf10 probe was a 584-bp fragment (nucleotides 11-579) inserted into BlueScript KSII+ vector.

**Preparation of embryonic lung tissue**

Embryonic lung dissection and epithelial and mesenchymal cell separation was performed as previously described (del Moral and Warburton, 2010), with small modifications. Briefly, pregnant mice were killed to harvest embryos at E11.5, E13.5 and E15.5 by CO<sub>2</sub> administration. Collected embryos were dissected under a stereoscopic microscope in a glass Petri dish immersed in PBS. Isolated lungs were then transferred to 24-well plates.
A quantitative PCR analysis

Normal adult lung tissue was homogenized using a Precellys homogenization kit (Bertin Technologies, Montigny-le-Bretonneux, France). Total RNA was extracted using NucleoSpin RNA II kit (MACHERY-NAGEL, Düren, Germany) and quantified using NanoDrop 1000 (Thermo Fisher Scientific Inc.). Complementary DNA (cDNA) was synthesized from the extracted RNA using a High-capacity cDNA reverse transcription kit (Applied Biosystems by Life Technologies/Thermo Fisher Scientific Inc.). q-PCR primers were designed to flank exon-exon boundaries and to give specific amplification. Following 3

Δ

Cq expression values

References


